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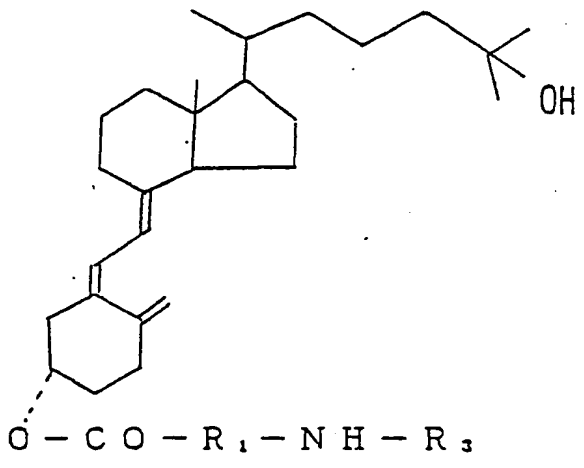
(54) 25-Hydroxy vitamin D3 derivatives.

(57) An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III):



wherein R_1 is a C_{1-10} alkylene group and R_3 is a group containing an iodine radioisotope, and the corresponding non-iodine radioisotopically labelled compound wherein R_3 is replaced by a hydrogen atom.

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Description

25-HYDROXY VITAMIN D_3 DERIVATIVES

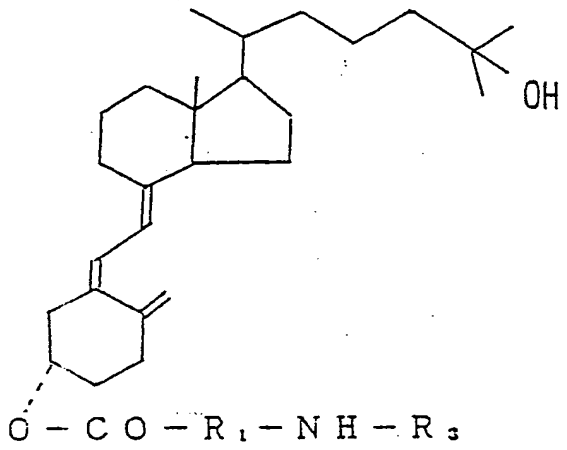
The present invention relates to 25-hydroxy vitamin D_3 derivatives, a process for their preparation and assay methods using them.

Known assay methods of 25-hydroxy vitamin D_3 include a competitive protein binding assay (CPBA) method and a radio immunoassay (RIA) method. In these methods, a tritium [^{3}H] labelled compound is used (PCT/JP 56/500538), for example a 25-hydroxy vitamin D_3 derivative labelled with tritium at the 1-position (JP-A-60-163859). In general the vitamin D binding protein used in the CPBA method is prepared from the plasma of a rat which has been fed with vitamin D deficient feed [Vitamin, 55(12), 595 - 605 : 1981]. A compound having a side chain with a carbonyl terminal group is known as a hapten for the preparation of antibodies used in the RIA method (JP-A-58-92656, JP-A-55-47653 and JP-A-59-148775). In these assay methods tritium labelled 25-hydroxy vitamin D_3 is used.

Compounds labelled with tritium have low specific activity as compared with those labelled with ^{32}P or ^{125}I in terms of radiation energy, and require high cost and cumbersome procedures. Known tritium labelled vitamin D_3 derivatives have only a low rate of β-ray emission. There have been no reports on ^{125}I-labelled vitamin D_3 derivatives, which would emit γ-rays with high energy, since the conjugated triene structure of vitamin D_3 is known to be unstable and thought to be subject to auto-degradation by a radical reaction.

We have found an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative with high radiation energy which has superior properties over tritium or ^{32}P-labelled compounds.

The present invention provides an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III):



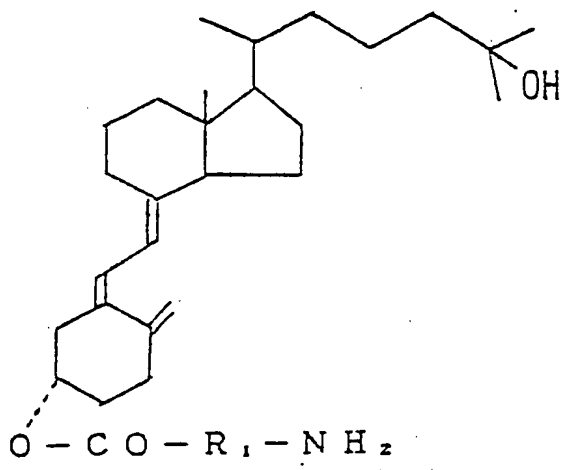
wherein R_1 is a C_{1-10} alkylene group and R_3 is a group containing an iodine radioisotope.

The iodine radioisotope is preferably ^{125}I. The vitamin D_3 derivative of the present invention is stable and not subject to structural degradation by γ-rays; hence it is useful for use in radio immunoassay techniques.

In general radioisotope labelling techniques, direct labelling (chloramin-T method) and indirect labelling (Bolton-Hunter reagent method) can be used to provide the radioisotope labelled compound (Amersham Note, 1 November 1981). The indirect labelling method with mild reaction conditions is preferred for preparing the derivative of formula (III) due to the instability of vitamin D against acids, oxygen, oxidizing agents, heat and light.

The derivative of formula (III) may be prepared from a 25-hydroxy vitamin D_3 amino acid derivative having a side chain terminated with an amino group.

The present invention therefore provides a 25-hydroxy vitamin D_3 amino acid derivative of formula (I):



wherein R_1 is a C_{1-10} alkylene, preferably a C_{2-6} alkylene, group.

The derivative of formula (I) may act as a hapten when it is bound to a carrier protein and inoculated into an animal to obtain antibodies. Using the antibodies and the iodine radioisotope labelled compound, we have also provided a highly sensitive and useful radio immunoassay system of 25-hydroxy vitamin D_3 in a specimen.

The present invention also provides an assay method of 25-hydroxy vitamin D_3 in a specimen which comprises:
adding, to a specimen containing 25-hydroxy vitamin D_3 to be assayed, an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III) as defined above;
adding anti 25-hydroxy vitamin D_3 antibodies to the specimen to bind competitively with the 25-hydroxy vitamin D_3 and the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III);
separating the thus produced iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative-anti 25-hydroxy vitamin D_3 antibody complex from the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative; and
measuring the amount of the iodine radioisotope label in bound or free form.

The iodine radioisotope labelled compound of the present invention can also be used in a CPBA method using DPB (vitamin D binding protein) for assaying 25-hydroxy vitamin D_3 with high sensitivity.

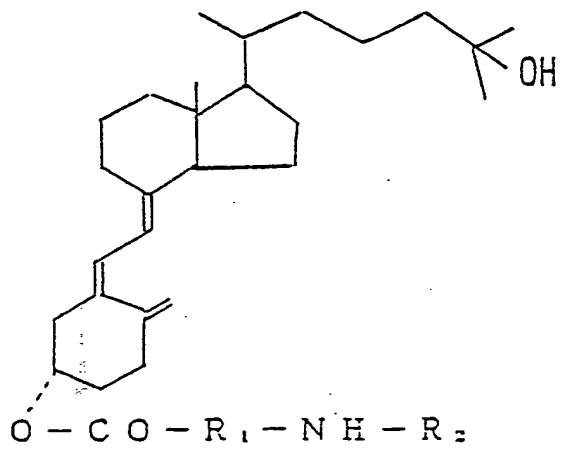
The present invention also provides an assay method of 25-hydroxy vitamin D_3 in a specimen which comprises:
adding, to a specimen containing 25-hydroxy vitamin D_3 to be assayed, an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III) as defined above;
adding vitamin D binding protein to the specimen to bind competitively with the 25-hydroxy vitamin D_3 and the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III);
separating the thus produced iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative-vitamin D binding protein complex from the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative; and
measuring the amount of the iodine radioisotope in bound or free form.

The 25-hydroxy vitamin D_3 amino acid derivative of formula (I) can be obtained by reacting an amino acid with the 3β-hydroxy group of 25-hydroxy vitamin D_3. The compound of formula (I) can be used as a hapten in a preparation of anti 25-hydroxy vitamin D_3 antibodies. Linkage of the hapten to the carrier protein is preferably by the C_{1-10} alkylene chain.

The compound of formula (I) can be prepared by reacting 25-hydroxy vitamin D_3 with an amino acid of formula (IV):

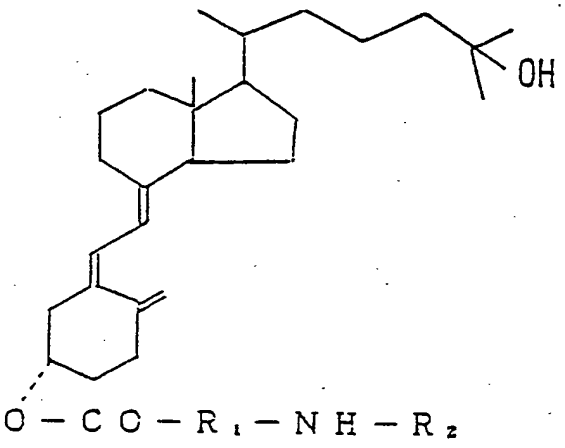
$HOOC\text{-}R_1\text{-}NH_2$

wherein R_1 is as defined above, the amino acid having a protected amino group, to obtain a 25-hydroxy vitamin D_3 derivative of formula (II):



and removing the protecting group therefrom.

The present invention also provides a process for preparing a 25-hydroxy vitamin D_3 amino acid derivative of formula (I) as defined above wherein a compound of formula (II):



wherein R_1 is as defined above and R_2 is an amino-protecting group, is deprotected in the presence of a base and in an inert solvent.

Examples of the number of carbon atoms in the R_1 group, and compounds from which they may be obtained, are: n = 1; glycine, n = 2; β-alanine, n = 3; γ-amino-butyric acid and β-amino-iso-butyric acid, n = 4; δ-amino valeric acid, n = 5; ε-amino-n-caproic acid, n = 6; 7-amino heptanoic acid, and n = 10; 11-amino undecanoic acid. The carbon chain in R_1 is preferably straight, and also preferably contains from 2 to 6 carbon atoms. δ-amino-levulinic acid, glycylglycine or δ-amino acid, ie D and/or L-lysine can also be used. An amino acid having a side chain hydroxyl, for example 4-amino-3-hydroxybutyric acid can be used if the hydroxyl group is protected. Thus the alkylene group represented by R_1 may be unsubstituted or substituted, for example by at least one amino, hydroxy or oxo group.

Suitable amino protecting groups are those which can easily be removed in mild conditions such as weakly basic conditions due to the possible instability of vitamin D under more severe removal conditions. Examples are 9-fluorenyl methyloxycarbonyl (hereinafter designated as Fmoc), 9-(2-sulfo)-fluorenyl methyloxycarbonyl, 1,1-dimethyl-2-cyanoethyloxycarbonyl and 5-benzisoxazolyl methyloxycarbonyl groups, most preferably a 9-fluorenyl methyloxycarbonyl group due to its ease in use. An amino-protected compound can be made by known methods by reacting an activated derivative, for example an activated ester of an Fmoc containing compound such as a 9-fluorenylmethyl-succinimidylcarbonate or 9-fluorenyl methylchloroformate derivative, with an amino acid [L.A. Carpino and G.Y. Ham. J. Org. Chem., 37: 3404 (1972)].

The 25-hydroxy vitamin D_3 amino acid derivative of formula (II) can be obtained, for example using Fmoc-amino acid as an amino protected amino acid, by reacting one equivalent of 25-hydroxy vitamin D_3 with one equivalent of Fmoc-amino acid or its acid anhydride, acid halide or activated ester. Preferably, one equivalent of Fmoc-amino acid is reacted with one equivalent of mixed anhydride having another acid under inert gas and base in anhydrous solvent. A reason for reacting the said mixed anhydride with 25-hydroxy vitamin D_3 is in order to prohibit a formation of a by-product, 25-hydroxy vitamin D_3 having a Fmoc-amino acid on a hydroxy group at postion-25.

Example of another acid in a mixed anhydride is valeric acid, pivalic acid or isobutylchloroformate and pivalic acid is preferable. Example of anhydrous solvent is anhydrous organic solvent such as tetrahydrofuran or dioxane. Example of base is preferably dimethylamino pyridine (DMAP) and piperidinopyridine (PPY) which are preferable for esterification of secondary alcohol or tertiary alcohol with steric hindrance. A preferable inert gas is argon or nitrogen. Reaction proceeds preferably at 0-20° C for 1-3 hours. The thus obtained 25-hydroxy vitamin D_3 derivative (II) can be purified, if necessary, by purification method such as column chromatography or thin layer chromatography (TLC).

The said 25-hydroxy vitamin D_3 derivative of the formula (II) is subjected to de-protection of amino group, ie removal of Fmoc, in the presence of base under inert solvent to produce 25-hydroxy vitamin D_3 derivative of the formula (I). Example of base is piperidine, morpholine or ethanolamine and morpholine is most preferable. Example of inert solvent is ethanol or methanol, preferably anhydrous ethanol or anhydrous methanol. Reaction proceeds under inert gas in a dark at 0 -20 °C for 1 - 3 hours.

The thus obtained 25-hydroxy vitamin D_3 derivative [I] can be purified by column chromatography or TLC.

In a radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative of the formula [III] , R_1 has the same meaning as of in the formula [I] and R_3 is radio isotope iodine labeled residue. Example of radio isotope iodine is ^{125}I or ^{131}I, and relatively long half-life isotope ^{125}I is preferable. Example of radio isotope iodine labeled residue is 3-(4-hydroxy-3-iodo [^{125}I] phenyl)-propionyl, 3-(3,5-diiod [^{125}I] -4-hydroxyphenyl) propionyl, 2-(4-hydroxy-3-iodo [^{125}I] phenyl) acetyl, 2-(3,5-di-iodo [^{125}I] -4-hydroxyphenyl) acetyl, 2-iodo [^{125}I] acetyl, 4-iodo [^{125}I] benzoxymethyl carbonyl or N-substituted-3-iodo [^{125}I] tyrosin residue. Among them 3-(4-hydroxy-3-iodo [^{125}I] phenyl) propionyl and 3-(3,5-diiodo [^{125}I] -4-hydroxyphenyl) propionyl are preferable.

In the production of radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative, 25-hydroxy vitamin D_3 amino acid derivative [I] is labeled with radio isotope iodine by indirect labeling method by means of Bolton-Hunter reagent to obatin a radio isotope iodine labeled 25-hydroxy vitamine D_3 derivative. An indirect labeling method herein is a method for producing radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative [III] by reacting a reactive derivative [IV] hereinbelow, which has a radio isotope iodine labeled residue, with 25-hydroxy vitamine D_3 amino acid derivative [I] .

The above reactive derivative [IV] is expressed by the formula

$$R_3 - X \quad [IV]$$

wherein R_3 has the same meaning hereinbefore, and X is succinimidyl-N-oxy, phthalimidyl-N-oxy, 5-norbornene-2,3-dicarboximidyl-N-oxy or maleimidyl-N-oxy. N-succinimidyl-3-(4-hydroxy-3-iodo [^{125}I] phenyl) propionate, for which R_3 is 3-(4-hydroxy-3-iodo [^{125}I] phenyl) propionyl and X is succinylimidyl-N-oxy in the compound [IV] , is a commercially available [^{125}I] Bolton-Hunter reagent. For example, indirect labeling method on 25-hydroxy vitamine D_3 amino acid derivative [I] using the said Bolton-Hunter reagent can be proceeded by reacting several p moles to several m mole of radio isotope iodine [^{125}I] Bolton-Hunter reagent with 500 ~2000 excess amount, preferably 1000 excess amount of 25-hydroxy vitamin D_3 amino acid derivative, at 0 - 30°C for 12 - 72 hours. In order to increase up an effect on RIA-or CPBA-method, the produced radio isotope labeled 25-hydroxy vitamin D_3 derivative [III] is preferably purified by TLC or HPLC. The thus obtained ^{125} I-labeled 25-hydroxy vitamin D_3 derivative is stable at -20° C in ethanol for more than two months of half-life of ^{125}I, and can be used in radio immunoassay.

Stock-temperature is preferable as low as possible at 5 °C ~ -20° C, preferably below -20° C, in alcohol or ether under inert gas.

Anti 25-hydroxy vitamin D_3 antibody can be prepared by inoculating a conjugation of a hapten, i.e. 25-hydroxy vitamin D_3 amino acid derivative [I] and a carrier protein into an animal. Examples of a carrier protein which is essential for obtaining an immunogenic antigen for hapten, are simple protein, polypeptide and complex protein such as glycoprotein. Example of simple protein is bovine serum albumin (BSA), human serum albumin or human serum globurin. Example of polypeptide is polylysine. Example of glycoprotein is mucoprotein. Among them simple protein is prefereable, especially bovine serum albumin and human serum albumin are most preferable.

25-hydroxy vitamine D_3 amino acid derivative [I] and a carrier protein is made on covalent bond in the presence of condensation reagent or crosslinkage reagent. Example of condensation reagent or crosslinkage reagent are dicyclohexylcarbodiimide (DCC), acid anhydride and glutaraldehyde, and DCC is most preferable. Conjugation ratio of 25-hydroxy vitamin D_3 amino acid derivative and carrier protein can be, due to cause decreasing titer of antibody if the one is excess, a bonding with 10 ~ 40 molecules of 25-hydroxy vitamin D_3 amino acid derivative is one molecule of carrier protein.

The thus prepared conjugate for antibody production is inoculated in an animal to produce antibody.

Inoculation can be made by parenteral administration such as subcutaneous or intracutaneous injection. A an inoculation, a conjugated antigen of the above 25-hydroxy vitamin D_3 amino acid derivative-carrier protein is dissolved in a buffer solution or physiological saline, together with adding equi-amount of complete Freund's adjuvant (C.F.A.), emulsifying the mixture completely, and inoculated subcutaneously or intracutaneously into a homeothermal animal, for about 10 times in every 1 ~ 3 weeks to imunize. Alternatively the conjugated antigen can be directly inoculated into a spleen. During an immunization period, serum antibody titer is measured in every constant times and at maximum titer whole blood sample is collected and allowed to stand for coagulation. Coagulated sample is centrifugally separated to obtain antiserum containing anti 25-hydroxy vitamin D_3 antibody.

Example of homeothermal animals is not limited and can be an animal which has antibody production activity, and is preferable to obtain large amount of antibody in sheep or bovine. In general rabbit or rat is used.

Isolation of anti 25-hydroxy vitamin D_3 antibody from antiserum can be made by conventional method for antibody purification. For example, ammonium sulfate fractionated antiserum is treated by ion-exchange chromatography or gel-filtration.

Another method of production of the antibody is that spleen cells, which can produce the desired antibody of animal, which is inoculated with conjugated antigen of 25-hydroxy vitamin D_3 amino acid derivative-carrier protein, are fused with established myeloma cells, and the thus obtained hybridoma is cultured, then monoclonal antibody which is produced by the said hybridoma is used.

For example, an emulsion, which is prepared by mixing a conjugated antigen of 25-hydroxy vitamin D_3 amino acid derivative-carrier protein dissolved in buffer solution or physiological saline and equall amount of C.F.A., is inoculated subcutaneously in mouse, for example Balb/c for sensitization. Cell-fusion is performed on 3 ~ 5 days after final sensitization. On 3 ~ 5 days after final sensitization, spleen cells which produce anti 25-hydorxy vitamin D_3 antibody are collected and fused with established myeloma cells which can be cultured for a long term. Long-term culturable established cell may be a cell which can be cultured and grown for long term in vitro or in vivo, which can produce immunoglobulin or its related protein, and generally well grown myeloma cells are used. Preferable examples of myeloma cells are cell-line of P3-NSI/1-Ag4-1, P3-X63-Ag8U1, SP2/U-Ag14 and MPC11-45.6.TG.1.7. In the present invention, P3-X63-Ag8U1 is preferable. The cells can be cultured in a conventional cell culture medium. For example culture can be performed in a medium of 10 % FCS added RPMI 1640 (Tradename, Flow Laboratory) which is added with glutamine, pyruvic acid, penicillin and streptomycin. For stock culture, S-azaguanine is added to the above medium. Myeloma cells, $1 \sim 3 \times 10^8$ cells, are used for a cell-fusion. Spleen cells can be prepared by cutting mouse spleen and crushing on a mesh to prepare spleen cell suspension. Washed cells, generally $1 \sim 3 \times 10^8$ cells, is fused with myeloma cells by mixing each cell. Ratio of mixing cells is experimentially myeloma cells : spleen cells 1 : 3 ~ 10. Cell-fusion is achived in a medium for hybridoma. In the cell-fusion, conventional cell-fusion method using promotor such as Sendai-virus or polyethylene glycol (PEG) is preferable. PEG is preferable in the present invention.

Fused cells are inoculated into the medium for hybridoma and incubated, then selected by incubating in HAT medium. HAT medium is a medium for hybridoma adding with hypoxanthine, aminopterin and thymidine.Since selected hybridoma is generally grown more than 2 hybridomas in a well of cell-separation plate, more than two kind of antibodies are possibly produced or no-antibody producing cells may be contaminated, so in order to obtain cells having same properties, each clone should be separated. For the cloning, a limiting dilution culture or soft agar culture is used. In this invention, limiting dilution culture is preferable.

The thus obtained hybridoma secreting anti 25-hydroxy vitamin D_3 antibody with high titer can be stocked by lyophilization at an early stage. Lyophilization can be made by a convnetional method, namely cell suspension in a small tube or ampule is freezed in -80 °C freezer and stocked in a liquid nitrogen. Another example of hybridoma production is that the above hybridoma is inoculated intraperioneally in pristan (2,6,10,14-tetramethylpentadecane, Aldrich Chemicals) treated mice, and after about 10 days ascites is collected. Further method is that the hybridoma is incubated in bovine fetal serum added RPMI medium or in Darbecco modified Eagle medium. Antibody thus obtained can be purified by a conventional method. For example antibody is fractionated with ammonium sulfate and treated by ion-exchange chromatographyhy, gel-filtration and affinity chromatography to fractionate IgG. Then purified anti 25-hydroxy vitamin D_3 monoclonal antibody can be obtained.

Furthermore, anti 25-hydroxy vitamin D_3 monoclonal antibody producing cells are inoculated and grown an animal having identical histocompatibility antigen or in nude mouse as a tumor, and grown cells are collected and the monoclonal antibody is purified therefrom.

In an assay of 25-hydroxy vitamin D_3, anti 25-hydroxy vitamin D_3 polyclonal antibody or anti 25-hydroxy vitamin D_3 monoclonal antibody (hereinafter sometimes totally designated as anti 25-hydroxy vitamin D_3 antibody) can be used in its soluble state or in an immobilized state. Insoluble carrier and anti 25-hydroxy vitamin D_3 antibody are bound by using polyfunctional reagent and the immobilized antibody has antibody titer against 25-hydroxy vitamin D_3. Example of polyfunctional reagent is a compound having more than two groups which can react with functional group such as amino, hydroxyl , carboxyl and thiol, and is aldehyes such as succinaldehyde, glutaradehyde or adipoaldehyde, dicarboxylate such as malonicacid, succiniacid glutaraiccid or adipicacid or its reactive derivative, diisocyanates such as hexamethylene diisocyanate or 2,4-toluenediisocyanate, diisothiocyanate such as hexamethylene diisothiocyanate, maleimide carboxylates such as maleimide benzonate or maleimide phenylacetate or its functional derivative, dimaleimides such as N,N-ethylene-bis-maleimide or N,N'-O-phenylene dimaleimide, bisdiazobenzidine, diethylmalonimidate, dimethyladipinimidate or

N,N'-polymethylene-bisiodo acetamid and thiocarboxylates such as 3-(2'-benzothiozolyl-dithio) propionate or 3-(2'-pyridyldithio) propionate or its functional derivative. The polyfunctional reagent can be selected by considering the bonding of functional group such as amino, carboxyl, hydroxyl or thiol in anti 25-hydroxy vitamin D_3 antibody. Immobilized carrier is a carrier having reactive group which does not bound with group for bonding with antibody in polyfunctional group. Examples of immobilized carrier are insoluble protein such as albumin or gelatin, epichlorhydrin treated insoluble polysacharide such as agarose, cellulose or dextrin, insoluble polymer or copolymer of acrylonitrile, acrylic acid, aclylate ester, metacrylic acid, metacrylate ester, vinylalcohol, vinylacetate, styrene, aminostyrene, chlorstyrene, maleic acid or fumaric acid, which is treated with bromocyanate and is introduced with spacer corresponding to amino group introduction, and insoluble inorganic carrier which is introduced with fnctional group such as amino group into inorganic compound such as silicon and aluminium. Immobilized carrier may also be a carrier which can bind anti 25-hydroxy vitamin D_3 antibody by physical adsorption.

Immobilized carrier is preferably be a particle which can easily isolated by filtration, for example beads having diameter more than 1 mm or preferably more than 5 mm, or a spindle form which corresponds to a bottom shape of antigen-antibody reaction tube.

Introduction of reactive group into anti 25-hydroxy vitamin D_3 antibody using spacer introducing reagent can be made by introducing additional functional group such as aldehyde, carboxyl, amino or thiol, reacting with one or more spacer introducing reagent, for example dialdehydes such as succinaldehyde, glutaraldehyde or adipoaldehyde, reactive derivative such as acid chloride, succinimide ester or p-nitrophenyl ester of ω-amino butyric acid or ω-amino glutamic acid, reactive derivative of dicarboxylic acids such as malonic acid, succinic acid, glutaric acid or adipic acid, diamines such as hexamethylene diamine or decamethylene diamine, reactive derivative of 3-(2'-pyridyl-dithio) propionic acid or 3-(2'-benzothiazolyl-dithio) propionic acid, S-acetylmercapto succinic anhydride or thiols such as 2-aminoethanethiol.

Anti 25-hydroxy vitamin D_3 antibody is condensed derectly or through polyfunctional reagent with reactive group in the immobilized carrier. Condensation reactive process generally at 0 - 40 °C in a pH 6.0 ~ 8.5 buffer solution or organic solvent or mixture thereof. Furthermore, a second antibody which is obtained by immunizing large mammals inoculated with immunoglobulin fraction in serum, which is used for antibody production of 25-hydroxy vitamin D_3, is immobilized and anti 25-hydroxy vitamin D_3 antibody is bound thereto by antigen-antibody reaction to prepare the immobilized antibody.

The thus obtained immobilized antibody is washed and stocked.

In an assay of 25-hydroxy vitamin D_3 in a specimen using anti 25-hydroxy vitamin D_3 antibody in a liquid soluble phase, a fixed amount of radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative [III] is added previously to a specimen containing 25-hydroxy vitamin D_3, then an optimum amount of anti 25-hydroxy vitamin D_3 antibody is added to form antigen antibody combining reaction product.

The thus formed labeled 25-hydroxy vitamin D_3 derivative-anti 25-hydroxy vitamin D_3 antibody binding complex, and 25-hydroxy vitamin D_3-antibody binding complex, and free labeled 25-hydroxy vitamin D_3 derivative are separated by using specific antibody for anti 25-hydroxy vitamin D_3 antibody. The said specific antibody is called as second antibody. The second antibody can be obtained, for example by inoculating normal immunoglobulin fraction in serum of animal which is used for antibody production of 25-hydroxy vitamin D_3, as an antigen to immunize, then isolatng from the thus obtained anti-serum. The second antibody can be purified if necessary by known method, or it can be preferably be used in the state of anti-serum.

An assay method of 25-hydroxy vitamin D_3 in a specimen using anti 25-hydroxy vitamin D_3 antibody and radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative is illustrated as follows.

25-hydroxy vitamin D_3 in a specimen such as known serum, is extracted from the serum specimen. A sample, which is a mixture of serum and equal amount of added solvent, is stirred, allowed to stand and centrifuged. Separated supernatant solution is treated by colum chromatography and collected a fraction of 25-hydroxy vitamin D_3. Further preferably the sample is purified by HPLC.

25-hydroxy vitamin D_3 fraction can be checked by previously added tritium [^{3}H] labeled 25-hydroxy vitamin D_3. The 25-hydroxy vitamin D_3 fraction is dried in vacuo, replaced by argon gas and dissolved in ethanol to prepare specimen. Fixed amount of radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative is added to a specimen, and the most suitable amount of anti 25-hydroxy vitamin D_3 antibody is added thereto. The mixture is incubated in a medium for antigen-antibody such as phosphate buffer or veronal buffer at 4 - 5° C for about 15 - 72 hours to proceed competitive reaction of radio isotope iodine labeled hapten and non-labeled hapten to antibody. The thus formed antigen antibody binding complex, namely a bound form of radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative-anti 25-hydroxy vitamin D_3 antibody (B) and an unreacted free form of radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative (F) are separated by dextran-charcoal (DC) method with filtration or centrifugation, at 3,000 r.p.m. for 15 minutes. On B-F separation, each ratio activity in B or F is measured.

Amount of 25-hydroxy vitamin D_3 (H) in a specimen is calculated by measuring radioactivity on B/(B +F) or B/F. Namely, when amount of H is increased radioactivity of B is decreased and that of F is increased. Therefore unknown amount of H can be obtained by measuring radioactivity of B and F from previously set up standard curve of known amount.

On the B-F separation, when double antibody technique is applied in a soluble state antibody, second antibody, preferably second antibody containing antiserum and if necessary normal serum of the same kind of animal used for anti 25-hydroxy vitamin D_3 antibody production are added after competitive reaction, and

incubated for 1 ~ 12 hours. Thereafter the formed binding complex is precipitated by centrifigation at 3,000 r.p.m. for 10 ~ 30 minutes to separate precipitate (B) and supernatant (F), then radio activity of B or F is measured.

According to the present assay method, standard curve on 2 pg/Test ~ 256 pg/Test can be prepared, and rapid reaction time of 16 hours at 5° C or 1 hour at 37° C can be achieved. Further operation after the reaction is quite simple. Moreover dilution and recovery test using specimen is shown as linearity with high precision. Correlation coefficient between [^{125}I] RIA method of the present invention and known [^{3}H] CPBA method on an assay of 25-hydroxy vitamin D_3 in human serum is : correlation coefficient $\gamma = 0.980$ ($y = 0.928X + 1.90$, $n = 18$).

Also when antibody is replaced by known DBP, it can be reacted with radio isotope iodine [^{125}I] labeled 25-hydroxyvitamin D_3 derivative and standard curve between 1 ~ 32 pg/Test can be prepared. The DBP can be obtained by serum of chicken, rat, mouse, rabbit, goat, sheep, bovine and human.

Following examples illustrate the present invention but are not construed as limiting.

Referential example 1:

Extraction and purification of 25-hydroxy vitamin D_3 in serum :

A mixture of serum 0.5 m ℓ and acetonitrile 0.5 m ℓ was stirred by BORTEX mixer then allowed to stand for 30 minutes. Supernatant solution 0.8 m ℓ obtained by centrifugation at 3,000 r.p.m. for 10 min. was charged on Sep-pack C - 18 cartridge column (tradename, MILLIPORE, Waters Corp.) which was activated with ethanol and equilibrated with 50% acetonitrile, and eluted with hydrated acetonitrile. Further column was washed with 50 % acetonitrile 4 m ℓ then eluted with 64 % acetonitrile 4 m ℓ (a fraction of 1 α,25-dihydroxyvitamin D_3), and eluted with 73 % acetonitrile 4 m ℓ (a fraction of 25-hydroxy vitamin D_3 and 24, 25, dihydroxyvitamin D_3). Fraction eluted with 73 % acetonitrile was dried up in vacuo, and packed with argon gas to obtain a fraction containing 25-hydroxy vitamin D_3. The thus obtained crude fraction was dissolved in a mixture 200 μ ℓ of n-hexane-isopropanol (9 : 1) purified by HPLC, Zorbax-SIL (Dupont Inc.) 0.46 × 25 cm column. The above fractions of 25-hydroxy vitamin D_3 was checked by previously added tritium [^{3}H] labeled 25-hydroxy vitamin D_3 [26, 27-methyl-^{3}H] . Fraction of 25-hydroxy vitamin D_3 (Rt = 4 ~ 5 min.) was dried up in vacuo and packed with argon gas.

The said fractions was dissolved in ethanol 2 m ℓ and 20μ ℓ thereof was used for an assay. Recovery of 25-hydroxy vitamin D_3 is 94.6 ± 2.4 % (n = 18).

Referential example 2:

3-(N-fluorenyl methyloxy carbonyl) amino propionic acid :

γ-amino-n-propionic acid (178 mg, 2mM) was added to N-succinimidyl-9-fluorenylmethyloxycarboxylate (674 mg, 2 mM) dissolved in a mixture of tetrahydrofuran (THF)-dimethylformamide (DMF)-H_2O (1 : 2 : 2) 25 m ℓ and reacted at room temperature for overnight. Reaction solvent was distilled off in vacuo and the residue was charged on a colum of silica-gel (Wako-gel C-200, 75 g) and separated and purified by eluting with $CHC\ell_3$: methanol = 9 : 1 to obtain compound 538.8 mg (yield : 86.5 %).

N M R. δ (D M S O - d_6)
2.50 - 2.60 (2H, t, -CH_2-Co-)
3.28 - 3.35 (2H, m, -CH_2-N-)
4.33 - 4.44 (3H, m, Fmoc)
7.35 - 8.06 (8H, m, Fmoc)

Example 1 :

25-hydroxy vitamin D_3-3β-0-3 [3'-(N-9-fluorenyl methyloxy carbonyl) aminopropionate] :

Pivaloyl chloride (7.38μ ℓ, d = 0.979, 0.06 mM) and dimethylamino pyridine (DMAP, 7.32 mg, 0.06 mM) were added to 3-(N-9-fluorenyl methyloxy carbonyl) amino propionic acid (18.7 mg, 0.06 mM) dissolved in dry THF 3mℓ, and reacted at -15° C for 15 minutes under argon atomsphare in a dark. THF solution 1 mℓ of 25-hydroxy vitamin D_3 (24.0 mg, 0.06 mM) was added thereto and reacted at 0 ° C for 1 hour and at room temperature for 1 hour, where 25-hydroxy vitamin D_3 was disappeared. Methanol 0.5 m ℓ was added to the mixture to stop the reaction, then distilled off in vacuo. Residue was purified by preparative TLC [Art. 5717, Merck, 20 × 20 cm, developer : ethylacetate-hexane (1 : 2)] to obtain the compound 30.1 mg (yield : 72.4 %).

N M R. δ ($CDC\ell_3$) p p m
0.537 (3H, s, CH_3-18)
3.30 - 3.60 (2H, m, -CH_2-N-)
4.10 - 4.50 (3H, m, Fmoc)
4.80 - 5.60 (4H, m, H-19E, H- 3α, H-19Z, -NH)
5.94 - 6.29 (2H, m, H-7, H-6)
7.20 - 7.81 (8H, m, Fmoc)
U V. λ_{max}^{EtOH} n m 300.2 266.4, 214.4

Preparation of radio isotope monoiodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative ; 25-hydroxy vitamin D_3- 3β-O - {3- [N-3-(4-hydroxy-3-iodo [^{125}I] phenyl) propionyl] amino propionate } :

Iodine [^{125}I] Bolton-Hunter reagent [NEN, NEX-120-10, 2200 Ci/mM, Total 0.33 m Ci/100μ ℓ Benzene solution] (100 pM/66.6 μ ℓ/220 μCi) and DMAP (1 mM/1 μ ℓ THF) were added to THF solution (130 μ ℓ) of 25-hydroxy vitamin D_3-3β-O-(3 -aminopropionate) (100 nM/127.8 μ ℓ THF) obtained in example 2, reacted at room temperature for 24 hours under argon atomosphere in a dark. Reaction mixture was purified by HPLC (Zorbax-SIL, 4.6 mm × 25 cm column, 20 % isopropanol-n-hexane, flow 1 mℓ/min.). The compound hereinabove has Rt = 8.50 - 9.50 min. and recovery 20.0 %. (Iodine [^{125}I] Bolton-Hunter reagent : Rt = 14.0 - 16.0 min.) Radio isotope monoiodine labeled 25-hydroxy vitamin D_3 was identified with synthesized non-radio isotope monoiodine labeled 25-hydroxy vitamin D_3 by HPLC.

Example 5 :

Preparation of radio isotope diiodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative ; 25-hydroxy vitamin D_3 -3β-O - {3- [N-3,5-diiodo-4-hydroxy [^{125} I] phenyl)propionyl] amino propionate} :

Iodine [^{125}I] Bolton-Hunter reagent (NEN, NEX-120H-10 , 4400 Ci/mM, Total 0.33 m Ci/100 μℓ Benzene solution] (25 pM/33.3 μ ℓ/110μCi) and DMAP (1 nM/1μ ℓ THF) were added to THF solution (120 μ ℓ) of 25-hydroxy vitamin D_3-3β-O-(3 -aminopropionate) (25 nM/6.952 μ ℓ THF) obtained in example 2, reacted at room temperature for 20 hours under argon atomosphere in a dark. Reaction mixture was purified by HPLC (Zorbax-SIL, 4.6 mm , × 25 cm column, 20 % isopropanol-n-hexane, flow 1 mℓ/min.). The compound hereinabove has Rt = 8.90 - 10.10 min. and recovery 9.9 %. (Iodine [^{125}I] Bolton-Hunter reagent : Rt = 14.0 - 16.0 min.) Radio isotope di-iodine labeled 25-hydroxy vitamin D_3 was identified with synthesized non-radio isotope di-iodine labeled 25-hydroxy vitamin D_3 by HPLC.

Example 6 :

Evaluation of radio isotope iodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative :

(1) Preparation of standard curve using radioimmuno assay of 25-hydroxy vitamin D_3 :

Standard ethanol solution of 25-hydroxy vitamin D_3 256 pg/20 μ ℓ was diluted seriesly in double in double dilution to prepare 128 pg/20 μ ℓ, 64 pg/ μ ℓ, 32 pg/20 μ ℓ ,16 pg/20μ ℓ, 8pg/20 μ ℓ, 4 pg/20 μ ℓ and 2pg/20 μ ℓ.

Samples (20 μ ℓ) of each concentration hereinabove were pipetted into 3 tubes. Ethanol solution, each 20 μ ℓ, of radio isotope iodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative which contains antioxidant tocopherol was pipetted into each tubes (20 μ ℓ ca. 13,000 cpm).

Anti 25-hydroxy vitamin D_3 rabbit serum diluted with Tris buffer (approx. 15,000 dilution) pH 8.6, each 1 m ℓ, was added in each tube. Each mixture in the tubes was stirred by BORTEX mixture and allowed to stand at 5 °C for overnight (20 - 24 hours). Glycine buffer, pH 8.6 suspended with a mixture of dextran T70 and active charcoal (1 : 10), (each 300 μ ℓ) was added into the reaction tubes, stirred by Bordex mixer and incubated at 5 °C for 1 hour. B-F separation was made by centrifugation at 3,000 r.p.m. for 10 minutes, and each supernatant solution 1 m ℓ was measured by autowell γ-counter for 1 min. A ratio of binding is caluculated by the following equation.

$$\text{Binding ratio} = \frac{(B) - (BL)}{(Bo) - (BL)} \times 100$$

wherein (B) is a count No. in each tube, (BL) is a count No. measured by using 25-hydroxy vitamin D_3 (1,000 pg/20μ ℓ) in place of isotope ^{125}I labeled 25-hydroxy vitamin D_3 derivative, and (Bo) is a count No. measured by using 25-hydroxy vitamin D_3 at zero concentration. Fig.1 shows standard curve of [^{125}I] labeled 25-hydroxy vitamin D_3 derivative.

(2) Comparison with tritium [^{3}H] labeled and [^{125}I] labeled 25-hydroxy vitamin D_3 derivative :

Standard curve was prepared by using tritium [^{3}H] labeled 25-hydroxy vitamin D_3 derivative (26, 27-methyl-^{3}H) as same as of in [^{125}I] labeled in (1) hereinabove.

Result is shown in Fig. 2 In Fig. 2, - O- O-: [^{125}I] labeled and - Δ- Δ-: [^{3}H] labeled compound were used . Equal or higher sensitivity was found when using [^{125}I] labeling as compared with [^{3}H] labeling, especially at low concentration (10 pg/Tube) higher sensitivity was observed.

Example 2 :

25-hydroxy vitamin D_3- 3 β-O-(3-aminopropionate):

25-hydroxy vitamin D_3-3β-O- [3-(N-9'-fluorenyl methyloxy carbonyl) aminopropionate] (30.1 mg, 0.043 mM) obtained in example 1 was dissolved in ethanol 1 mℓ. Morpholine 10 mℓ was added thereto and stirred at room temperature for 1 hour, under argon atomosphere in a dark to complete the reaction. Reaction mixture was concentrated in vacuo and purified by preparative TLC [Art. 5717, Merck, 10 × 20 cm, developer : ethyl acetate-MeOH (1 : 4)] to obtain the compound 8.3 mg (yield : 40.6 %).

Ninhydrin colorization : positive

N M R. δ (C D C ℓ_3) p p m

0.54 (3H, s, CH_3-18)

2.36 - 2.52 (2H, m, -CO-CH_2-)

2.90 - 3.05 (2H, m, -CH_2-N-)

3.50 - 4.00 (2H, b, -NH_2)

4.86 - 5.08 (3H, m, H-19E, H- 3α,H-19Z)

5.90 - 6.30 (2H, m, H-7, H-6)

U V. λ_{max}^{EtOH} n m 264.5

Example 3 :

25-hydroxy vitamin D_3- 3 β-O - [3 -(BSA-amino) propionate] antigen and : 25-hydroxy vitamin D_3 antiserum :

(1) Preparation of 25-hydroxy vitamin D_3- 3β-O- [3-(BSA-amino) propionate] antigen.

25-hydroxy vitamin D_3 -3 β-O- [3 -aminopropionate (18.1 mg, 38.43 × 10^{-3} mM) obtained in example 2 was dissolved in THF 1 mℓ, and was added to BSA (M.W. 65,000, 50 mg, 1/50 × 38.43 × 10^{-3} mM) dissolved in Tris-buffer (0.1 M, pH 8.6) at 0°C with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (9.6 mg,1.3 × 38.43 × 10^{-3} mM). Chloroform 10 m ℓ was added 4 times to the reaction mixture and unreacted 25-hydroxy vitamin D_3- 3β-O-(3 -aminopropionate ester) was removed off. Aqueous layer was freeze dried to obtain the lyophilized product 45 mg which was a complex of 22 molecules of 25-hydroxy vitamin D_3-3β-O-(3 -amino propionate ester) bound through amino group at position-3 thereof in one molecule of BSA.

(2) Preparation of 25-hydroxy vitamin D_3 antiserum :

Lyophilized 25-hydroxy vitamin D_3- 3 β-O- [3 -(BSA-amino) propionate] (antigen) obtained in the above (1) was dissoleved in Tris buffer (0.1 M, pH 8.6). Equal amount of C.F.A. was added thereto and mixed to emulsify to prepare antigen 1 μg ~ 200μg/mℓ. The emulsion was inoculated subcutaneously 10 times for every 2 weeks, 50γ - 500 γ/head, in rabbit. During immunization, titer of blood sample collected at every 10 days was measured and at maximum antibody titer whole blood was collected. Blood sample was allowed to stand at room temperature for 60 minutes to coagurate and centrifuged at 3,000 r.p.m. for 10 min. to obtain antiserum containing anti 25-hydroxy vitamin D_3 antibody, which was fractionated with ammonium sulfate to collect IgG fraction.

(3) Preparation of anti 25-hydroxy vitamin D_3 monoclonal antibody :

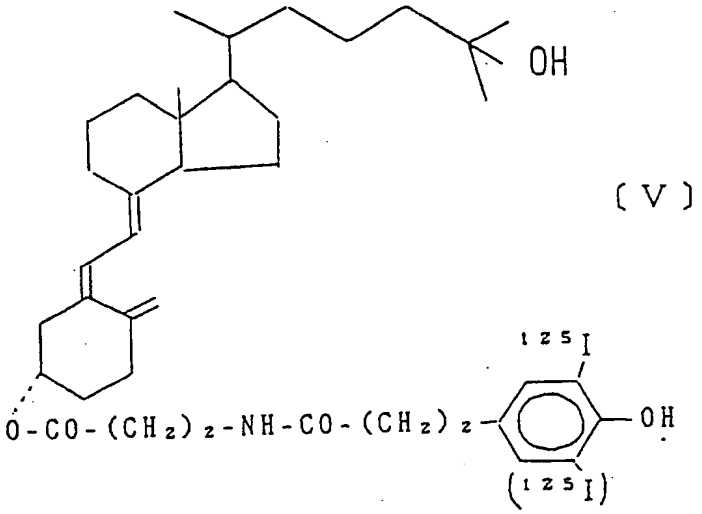
Lyophilized 25-hydroxy vitamin D_3-3β-O- [3 -(BSA-amino) propionate] (antigen) obtained in the above (1) dissolved in phosphate buffer, pH 7.2, 50 γ together with C.F.A. was inoculated subcutaneously in Balb/c mice, female, 4 weeks age. After 1 week 50γ thereof was subcutaneously inoculated and after 2 weeks 50 γ of antigen was intraperitoneally inoculated. On 3 days after the final inoculation, spleen was finely cut and crushed on the mesh to prepare spleen cell suspension. Cell fusion was performed using mouse myeloma cell P3-X-3-Ag8U1 by a conventional method. 30 % PEG (M.W. 1,000) aqueous solution was incubated at 37 °C. Spleen cells hereinabove and myeloma cells (total 4 × 10^7 cells, 5 : 1) were suspended in RPMI medium (5 m ℓ) and both cells were gently mixed, centrifuged at 1,000 r.p.m. for 10 minutes, then the supernatant was vacuum filtred. Test tube was weaky shaked to mix cell pellets and PEG solution 1.0 mℓ was slowly added and gently stirred. Mixture was incubated at 37 °C with gently shaken, then fusion reaction was stopped by adding slowly conventional medium (10 m ℓ) and again cells curve suspended. Suspension was centrifuged at 1,000 r.p.m. for 5 minutes. Precipitated cell pellet was dispersed by gently shaking and slowly suspended in HAT medium (5 mℓ) and was transferred into HAT-medium (1m ℓ) in a vessel. Cells were observed microscopically. Cell suspension was pipetted each 200 μ ℓ into a well in 96 well plate and, grown in the CO_2-incubator. Fused cells were selected in HAT medium to cloning by limiting dilution technique. The obtained clone suspension was grwon in a peritoneal of pristan treated Balb/c mouse. IgG fraction was collected by conventional method from ascites and serum and purified by affinity chromatography using protein A bound Sepharose CL-4B. The thus obtained IgG sub-class was IgG 1 which was used as monoclonal antibody.

Example 4 :

(3) Comparison with mono-iodine [^{125}I] labeling and di-iodine [^{125}I] labeling :

Mono-iodine [^{125}I] labeling and di-iodine [^{125}I] labeling were compared according to the same method hereinabove. Result is shown in Fog. 3. In Fig. 3, -●- ●-: mono-iodine [^{125}I] labeling, and - ■ - ■ - : di-iodine [^{125}I] labeling.

Radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative is illustrated in the formula [V]



Assay using mono-iodine [^{125}I] labeled compound and di-iodine [^{125}I] labeled compound shown almost same sensitivity, especially mono-iodine [^{125}I] labeling is preferable.

(4) Comparison with methylene chain length in R_1 :

In the formula [IV] , assay using a compound having methylene R_1 [-(CH_2)n-] wherein n = 1, n = 2 or n = 3, is compared with each other.

Result is shown in Fig. 4. in which ▲- ▲- : n = 1, - ●- ●- : n = 2 and - ■ - ■ - : n = 3. The n = 2 and n = 3, preferably n = 2 are shown in good result as compared with n = 1.

Example 7 :

Evaluation of radio isotope iodine labeled 25-hydroxy vitamin D_3 by CPBA method using DBP in place of anti 25-hydroxy vitamin D_3 antibody :

(1) Preparation of DBP :

Fetal calf serum (FCS) 3.3 m ℓ diluted with phosphate buffer (pH 7.4, 0.01M) was charged on a column of Blue-Spharose (Tradename Pharmacia Corp. φ 2.5 × 7.5 cm) and de-albumin treatment through gel-bed volume 40 m ℓ was performed.

Fractions, each 5 m ℓ, No. 7 - 10, checking with absorption at 280 nm were collected (total 20 m ℓ). Instead, FCS can be replaced by serum of homeothermal animals.

(2) Standard curve of radio isotope iodine [^{125}I] labeled 25-hydroxy vitamin D_3 by CPBA-method using DBP, replaced for antibody :

Standard ethanol solution of 25-hydroxy vitamin D_3 1.024 pg/20 μ ℓ was diluted seriesly in double in double dilution to prepare 32 pg/20 μ ℓ, 16 pg/20 μ ℓ, 8 pg/20 μ ℓ , 4 pg/20 μ ℓ, 2 pg/20 μ ℓ and 1pg/20 μ ℓ.

Samples (20 μ ℓ) of each concentration hereinabove were pipetted into 2 tubes. Ethanol solution, each 20 μ ℓ, of radio isotope iodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative which contains antioxidant tocopherol was pipetted into each tubes (20 μ ℓ ca. 30,000 cpm). DBP diluted with Tris buffer, pH 8.6 (approx. 500 ~ 1,000 dilution of DBP fraction in (1) hereinabove) each 500 μ ℓ, was stirred and allowed to stand at 4 °C for 16 hours.

Glycine buffer, pH 8.6 suspended with a mixture of dextran T70 and active charcoal (1 : 10), (each 300 μ ℓ) was added into the reaction tubes, stirred and incubated at 4 °C for 30 minutes. B-F separation was made by centrifugation at 3,000 r.p.m. for 10 minutes at 4°C, and each supernatant solution 500 μ ℓ was measured by autowell γ-counter for 1 min. A ratio of binding is caluculated by the following equation.

$$\text{Binding ratio} = \frac{(B) - (NSB)}{(Bo) - (NSB)} \times 100$$

wherein (B) is a count No. in each tube, (NSB) is a count No. measured by using 25-hydroxy vitamin D_3 (1024 pg/20 μℓ) instead of ^{125}I labeled 25-hydroxy vitamin D_3 derivative and (Bo) is a count No. measured by using 25-hydroxy vitamin D_3 at zero concentration. Fig. 5 shows standard curve of [^{125}I] labeled 25-hydroxy vitamin D_3 derivative.

(3) Stability of radio isotope iodine [^{125}I] labeled 25-hydroxy vitamin D_3 derivative :
50 % H_2O-ethanol solution of [^{125}I] 25-hydroxy vitamin D_3 derivative obtained in example 4 or example 5 was stocked at -20°C, in argon atomosphere in vial. Standard curve using the avove sample was prepared according to a method (2) hereinabove. In Fig. 6 -○-○- : day 0, -Δ-Δ- : day 21 and -□-□- : day 49 of standard curve are shown. As shown in Fig. 6, when it was stored at -20 °C, no changes of standard curve within a range of 2 ~ 32 pg/Test were observed. The result shows that the [^{125}I] 25-hydroxy vitamin D_3 derivative can be used in radio immunoassay with good stability.

Effect of invention :

As illustarated hereinabove, the present invention provides radio isotope iodine labeled 25-hydroxy vitamin D_3 derivative with high radiation energy and widely usability. Heretofore, low radiation energy, β-ray emission tritium [^3H] labeled vitamin D_3 has been known. Until now, high radiation energy radio isotope iodine [^{125}I] vitamin D_3 is thought to degradate automatically due to deriving radical reaction on conjugated double bond in vitamin D structure. [^{125}I] labeled 25-hydroxy vitamin D_3 derivative of the present invention does not derive autodegradation by γ-ray and is quite stable for use in radio immunoassay.

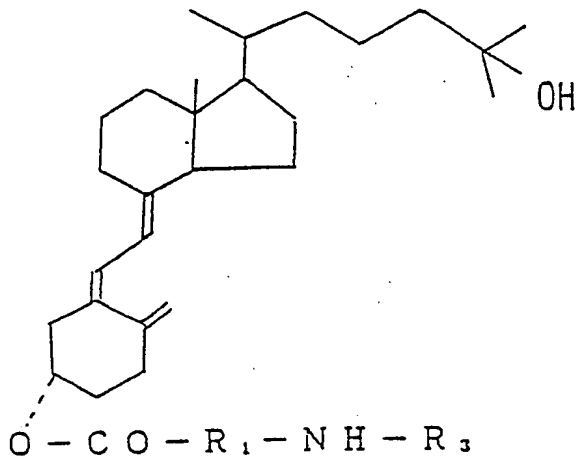
In general, vitamin D_3 is metabolized in vivo in liver or kidney and converted to activated Vitamin D_3. The activated vitamin D_3, such as 25-hydroxy vitamin D_3, 1α, 25-dihydroxy vitamin D_3, 1α-hydroxy vitamin D_3 and 1 α,24 -dihydroxy vitamin D_3, is used clinically for the therapy of osteoporosis and osteomalacia. The clinical dose thereof is quite low due to its strong physicological action. Pharmacological activity is correlated to blood level and tissue level of drug and hence measurement of blood level of drug administered in human is important in clinical point of view. Considering the above the present invention is valuable in an assay of activated vitamin D_3 in clinical trials.

Brief explanation of drawings :

Fig.1 : Standard curve of [^{125}I] labeled 25-hydroxy vitamin D_3 derivative ;
Fig. 2 : Standard curve of [^{125}I] or [^3H] labeled 25-hydroxy vitamin D_3 derivative ;
Fig.3 : Standard curve of mono-iodo [^{125}I] or di-iodo [^{125}I] labeled 25-hydroxy vitamin D_3 derivative ;
Fig.4 : Standard curve of compound [IV] wherein R_1 [-(CH_2)n-] , n = 1, n = 2 or n = 3 ;
Fig.5 : Standard curve of [^{125}I] labeled 25-hydroxy vitamin D_3 derivative on CPBA-method using DBP instead of anti 25-hydroxy vitamin D_3 antibody ; and
Fig.6 : Stability curve of [^{125}I] labeled 25-hydroxy vitamin D_3 derivative on CPBA-method using DBP instead of anti 25-hydroxy vitamin D_3 antibody.

Claims

1. An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III):



wherein R_1 is a C_{1-10} alkylene group and R_3 is a group containing an iodine radioisotope.

2. An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative according to claim 1 wherein the iodine radioisotope is ^{125}I.

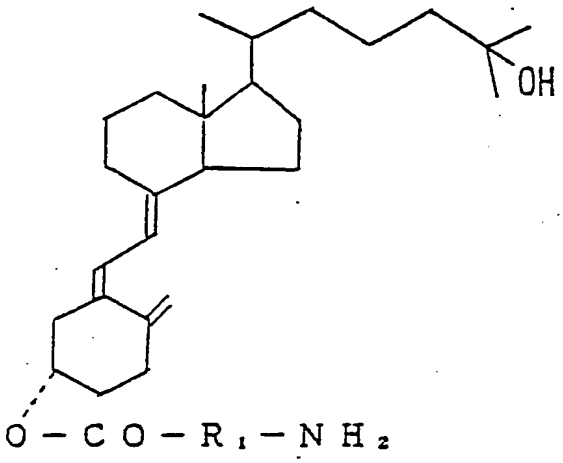
3. An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative according to claim 2 wherein the group containing an iodine radioisotope is a 3-(4-hydroxy-3-iodo[^{125}I]-phenyl)-propionyl or 3-(3,5-dilodo[^{125}I]-4-hydroxyphenyl)-propionyl group.

4. An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative according to any one of claims 1 to 3 wherein R_1 is a C_{2-6} alkylene group.

5. An assay method of 25-hydroxy vitamin D_3 in a specimen which comprises:
adding, to a specimen containing 25-hydroxy vitamin D_3 to be assayed, an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III) as defined in any one of claims 1 to 4;
adding anti 25-hydroxy vitamin D_3 antibodies to the specimen to bind competitively with the 25-hydroxy vitamin D_3 and the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III);
separating the thus produced iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative-anti 25-hydroxy vitamin D_3 antibody complex from the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative; and
measuring the amount of the iodine radioisotope label in bound or free form.

6. An assay method of 25-hydroxy vitamin D_3 in a specimen which comprises:
adding, to a specimen containing 25-hydroxy vitamin D_3 to be assayed, an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III) as defined in any one of claims 1 to 4;
adding vitamin D binding protein to the specimen to bind competitively with the 25-hydroxy vitamin D_3 and the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III);
separating the thus produced iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative-vitamin D binding protein complex from the iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative; and
measuring the amount of the iodine radioisotope in bound or free form.

7. A 25-hydroxy vitamin D_3 amino acid derivative of formula (I):



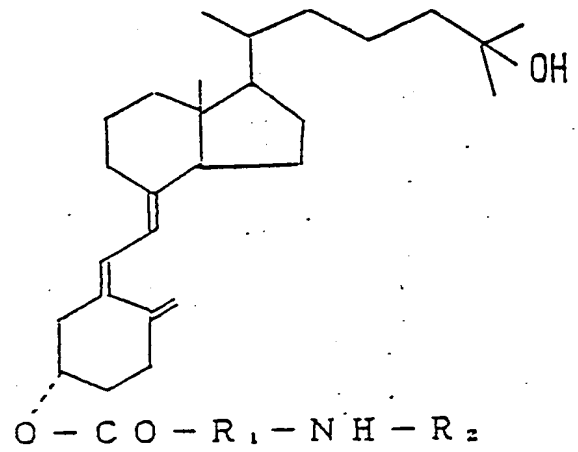
wherein R_1 is as defined in claim 1 or 4.

8. A process for preparing an iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III) as defined in any one of claims 1 to 4 wherein a 25-hydroxy vitamin D_3 derivative of formula (I) as defined in claim 7 is reacted with a compound of formula (IV):

R_3-X

wherein R_3 is as defined in any one of claims 1 to 3 and X is a succinimidyl-N-oxy, phthalimidyl-N-oxy, 5-norbornene-2,3-dicarboximidyl-N-oxy or maleimidyl-N-oxy group.

9. A process for preparing a 25 hydroxy vitamin D_3 amino acid derivative of formula (I) as defined in claim 1 wherein a compound of formula (II):



wherein R_1 is as defined in claim 1 or 4 and R_2 is an amino-protecting group, is deprotected in the presence of a base and in an inert solvent.

10. A process according to claim 9 wherein R_2 is a 9-fluorenyl methyloxycarbonyl group.

FIG. 1

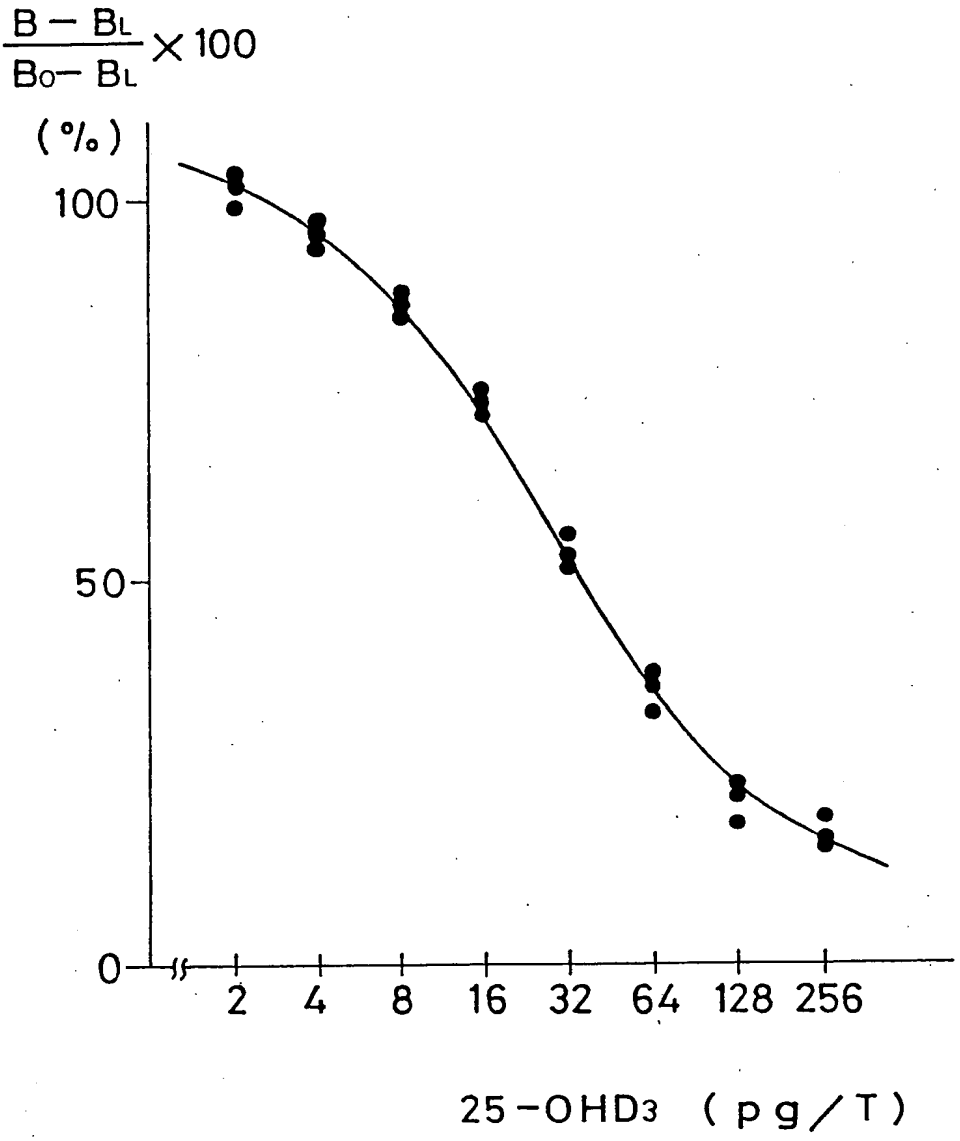


FIG. 2

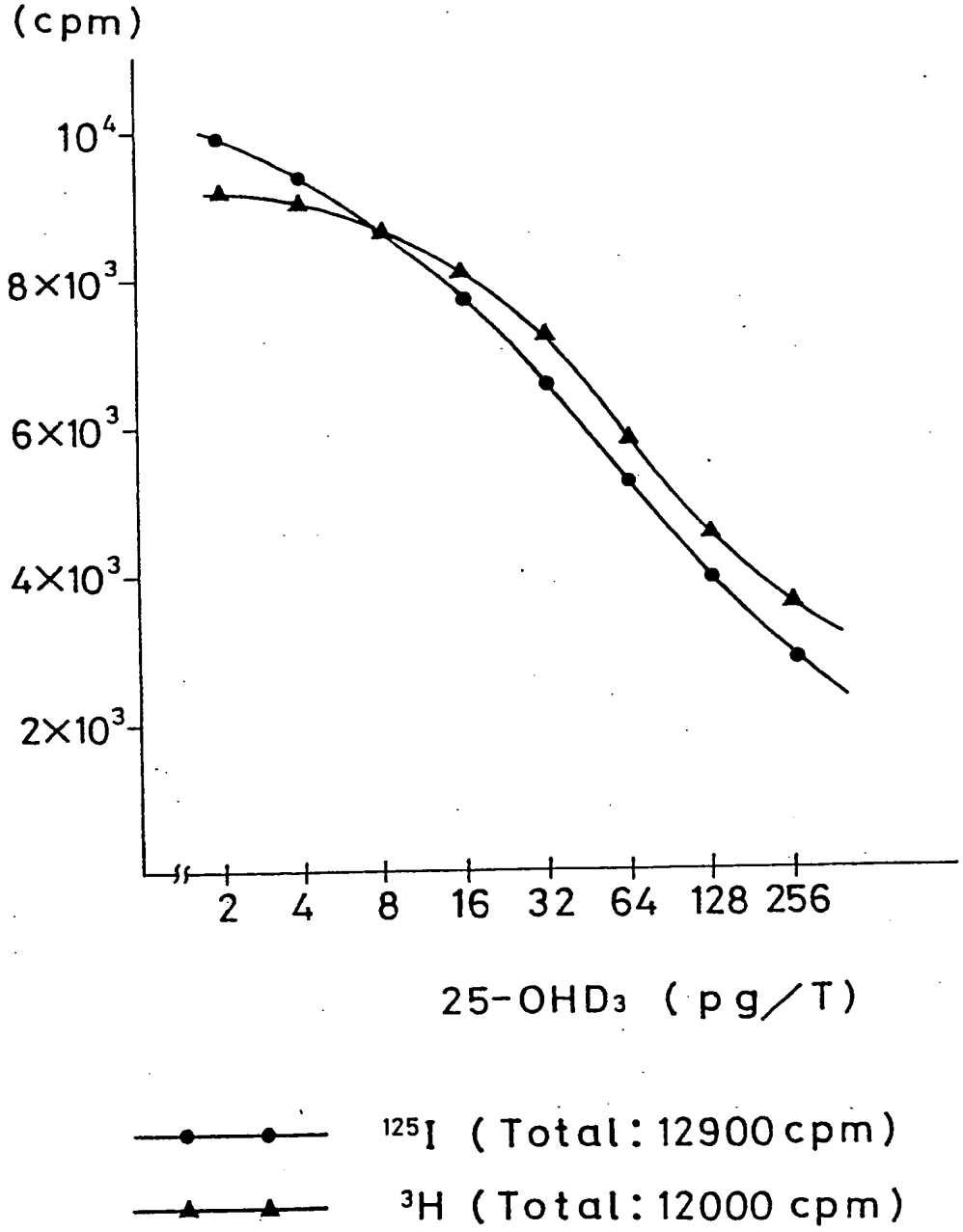

(cpm)
10^4
8×10^3
6×10^3
4×10^3
2×10^3
2
4
8
16
32
64
128
256
25-OHD3 (pg/T)
^{125}I (Total: 12900 cpm)
^{3}H (Total: 12000 cpm)

FIG. 3

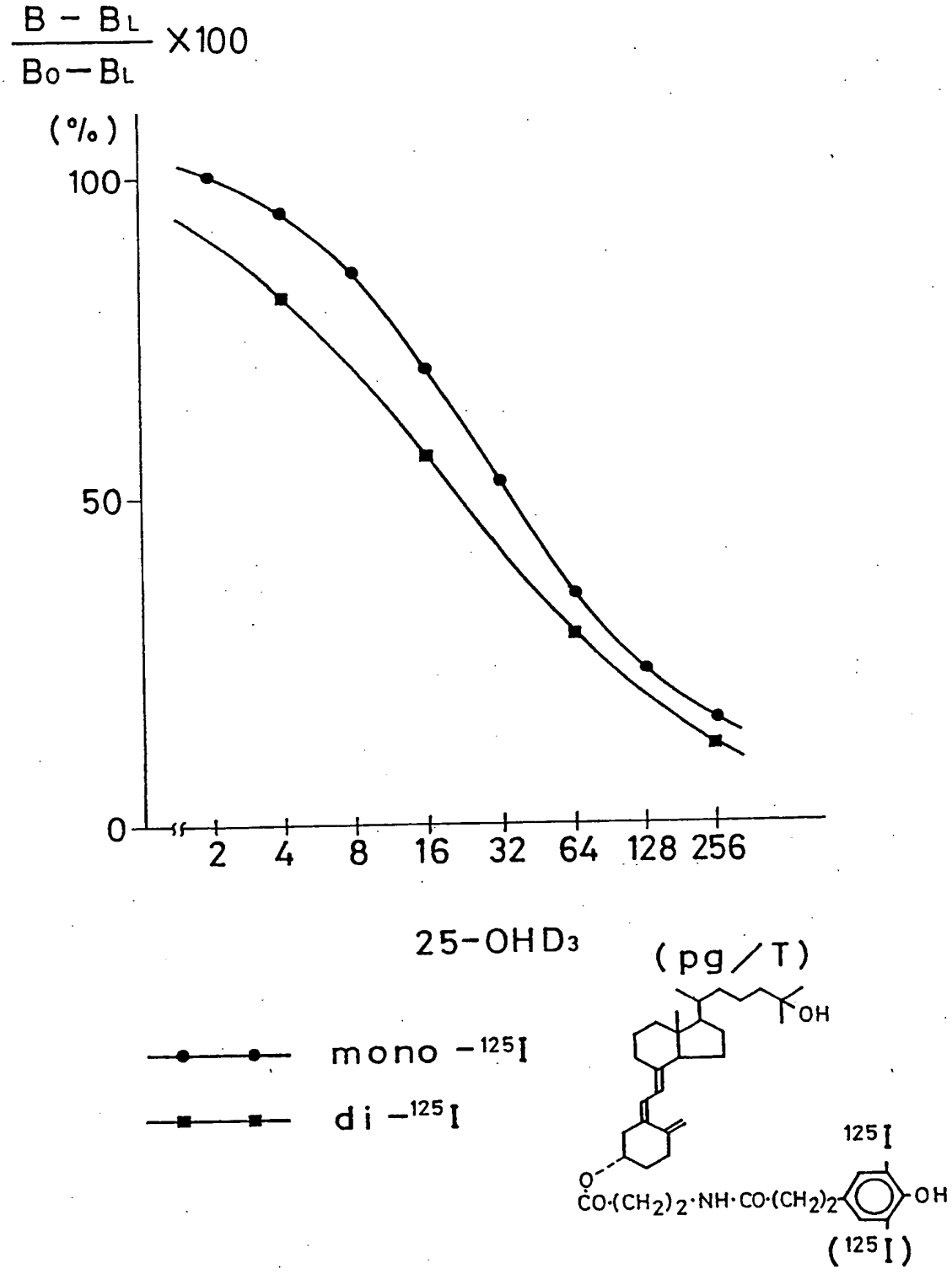

B − BL / Bo − BL X100
(%)
100
50
0
2
4
8
16
32
64
128
256
25-OHD3
(pg/T)
mono −125I
di −125I
OH
125I
O
CO·(CH2)2·NH·CO·(CH2)2
OH
(125I)

FIG. 4

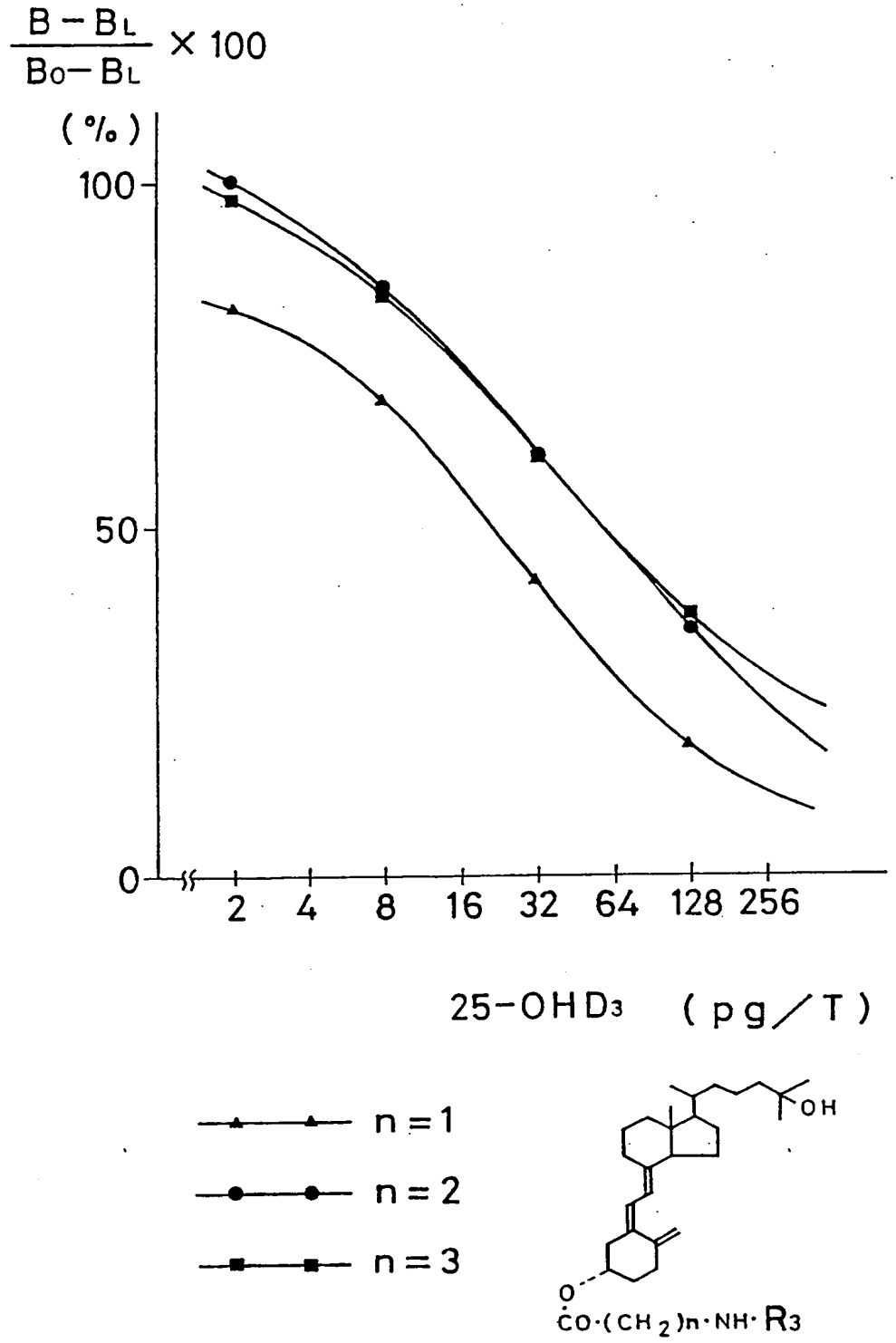

B − BL / Bo − BL × 100
(%)
100
50
0
2
4
8
16
32
64
128
256
25-OHD3 (pg/T)
n = 1
n = 2
n = 3
OH
O
CO·(CH2)n·NH·R3

FIG. 5

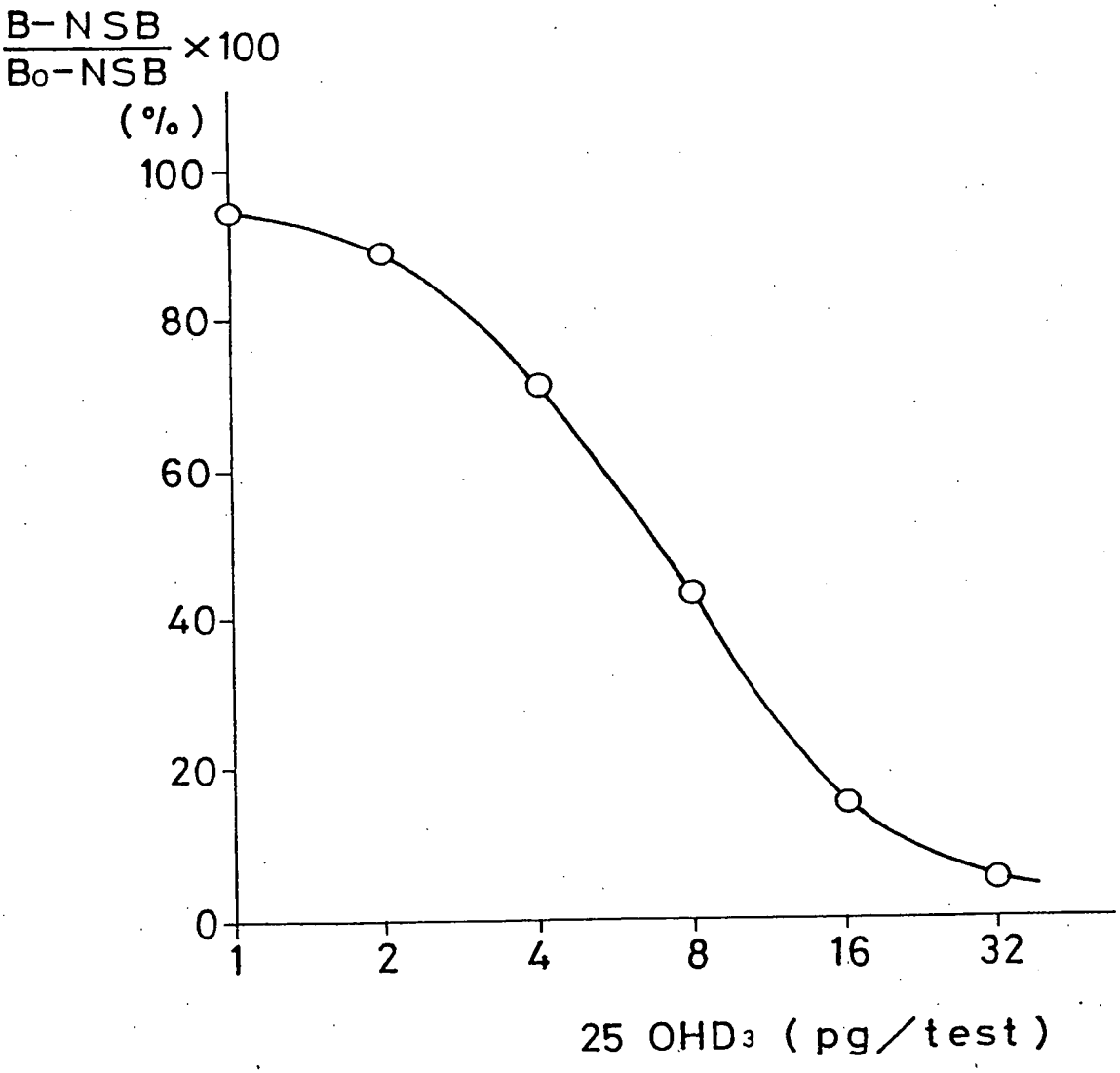
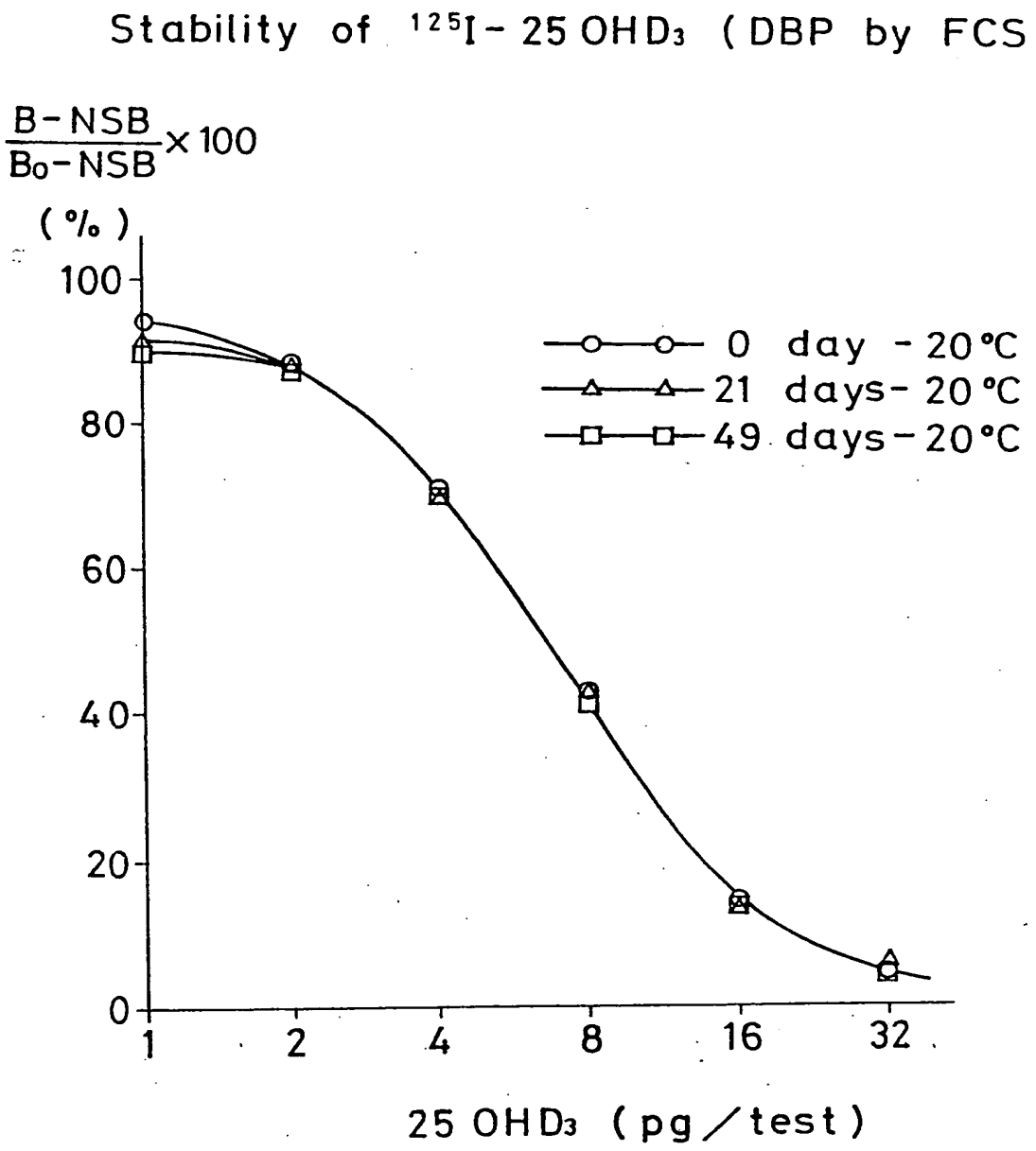


FIG. 6


Stability of ^{125}I-25 OHD$_3$ (DBP by FCS)
$\frac{B-NSB}{B_0-NSB} \times 100$
(%)
100
80
60
40
20
0
1
2
4
8
16
32
0 day -20°C
21 days-20°C
49 days-20°C
25 OHD$_3$ (pg/test)

(19) Europäisches Patentamt
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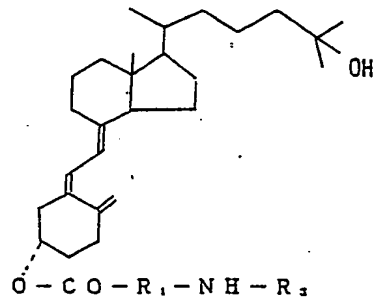
(54) 25-Hydroxy vitamin D3 derivatives.

(57) An iodine radioisotope labelled 25-hydroxy vitamin D_3 derivative of formula (III):



wherein R_1 is a C_{1-10} alkylene group and R_3 is a group containing an iodine radioisotope, and the corresponding non-iodine radioisotopically labelled compound wherein R_3 is replaced by a hydrogen atom.

EP 0 312 360 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 30 9609

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	JOURNAL OF BIOCHEMISTRY, vol. 98, no. 4, October 1985, pages 991-998, Tokyo, JP; I. YAMAMOTO et al.: "Monoclonal antibody for calcitriol (1alpha,25-dihydroxyvitamin D3)" * Whole article *	1,5-7	C 07 C 172/00 C 07 B 59/00 G 01 N 33/82
A	FR-A-2 376 864 (F. HOFFMANN-LA ROCHE) * Claims *	1,5,6	
A	US-A-4 424 161 (M.F. HOLICK) * Whole document *	1,5,6	
A	US-A-4 119 647 (A.A. LIEBMAN) * Whole document *	1,5,6	
A	WO-A-8 002 562 (WISCONSIN ALUMNI) * Claims *	1,5,6	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 07 C 172/00
C 07 B 59/00
G 01 N 33/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	01-08-1989	HENRY J.C.

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